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Microtubule-Dependent Redistribution of a Cytoplasmic Cornified Envelope Precursor

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Several cytoplasmic cornified envelope precursors have been described. Nevertheless, the mechanism whereby these proteins are positioned at the site of crosslink formation is not known. In this study, we examine the intracellular distribution of the cornified envelope precursor S100A11 (S100C) and the effects of the physiologic differentiating agent calcium on this distribution. S100A11 is localized in the cytoplasm of resting cultured human keratinocytes. Treatment with calcium causes S100A11 to relocate to the cell periphery. Immunoprecipitation studies reveal that S100A11 associates with microtubules, and inhibitor studies indicate that functional microtubules are required for S100A11 peripheral redistribution. Parallel studies indicate that S100A11 is not present in the Golgi or endoplasmic reticulum (ER), suggesting that S100A11 is not moved to the cell periphery via the classical Golgi/ER export pathway. Further evidence that the Golgi/ER is not involved is provided by the observation that the Golgi/ER disruptor brefeldin A does not alter movement. These results suggest that redistribution along microtubules is a mechanism whereby S100A11 is positioned at the cell periphery in preparation for transglutaminase-dependent crosslinking. Staining of epidermal tissue sections from uninvolved and psoriatic epidermis reveals strong staining at the cell periphery in the majority of suprabasal cells, confirming a peripheral distribution of S100A11 *in vivo*.

Key words: keratinocyte differentiation/S100C/calcium/tubulin/epidermis.
J Invest Dermatol 122:29–38, 2004

S100 proteins are small, multifunctional, calcium-regulated proteins (Donato, 1986; Heizmann, 1986; Schafer and Heizmann, 1996) encoded by genes that are clustered in the epidermal differentiation complex on chromosome 1q21 (Moog-Lutz *et al*, 1995; Mischke *et al*, 1996). These EF-hand-containing proteins undergo a conformational change in response to increased intracellular calcium (Donato, 1999). This conformation change exposes domains that permit S100 proteins to interact with target proteins to modify target protein function. S100A11 is a member of this family that is localized in the cytoplasm in resting keratinocytes. Our previous studies demonstrate that S100A11 serves as a transglutaminase substrate and a cornified envelope precursor (Robinson *et al*, 1997; Robinson and Eckert, 1998; Ruse *et al*, 2001).

The mechanism whereby cornified envelope precursors are positioned at the cell periphery is not well understood. Several envelope precursors, including loricrin and profilaggrin, are deposited at the site of envelope formation via vesicle delivery mechanisms (Kalinin *et al*, 2002; Steven *et al*, 1990). This occurs late in the envelope assembly process. Nevertheless, this vesicle-based mechanism cannot explain the positioning of soluble cytoplasmic envelope precursors at the cell periphery that occurs early in envelope assembly. It has been proposed that soluble proteins such as involucrin may move to the plasma membrane via a diffusion-based mechanism (Eckert *et al*, 1993; Kalinin *et al*, 2002); however, little *in situ* evidence is available to support this claim. In this report, we have selected S100A11 as a model protein to study the mechanism of soluble cornified envelope precursor movement to the cell periphery. Our results suggest that calcium promotes mobilization of S100A11 to the cell periphery and that the S100A11 travels along the microtubule network.

Abbreviations: ER, endoplasmic reticulum; HRP, horseradish peroxidase; PBS, phosphate-buffered saline.

Results

S100A11 antibody specificity We first assessed the specificity of the S100A11 antibody. Normal human keratinocytes were fixed and stained with anti-S100A11. As shown in Fig 1*A*, the antibody detected a diffuse pattern of cytoplasmic staining (anti-S100A11). In contrast, the signal is competed by incubation of the antibody with rhS100A11 (anti-S100A11 + rhS100A11). In addition, no signal is observed when a preimmune antibody is used or the primary antibody is omitted (not shown). Figure 1*B* shows that anti-S100A11 detects rhS100A11 (A11), but not the related S100 protein, rhS100A10 (A10), and Fig 1*C* shows that adsorption of anti-S100A11 with rhS100A11 eliminates

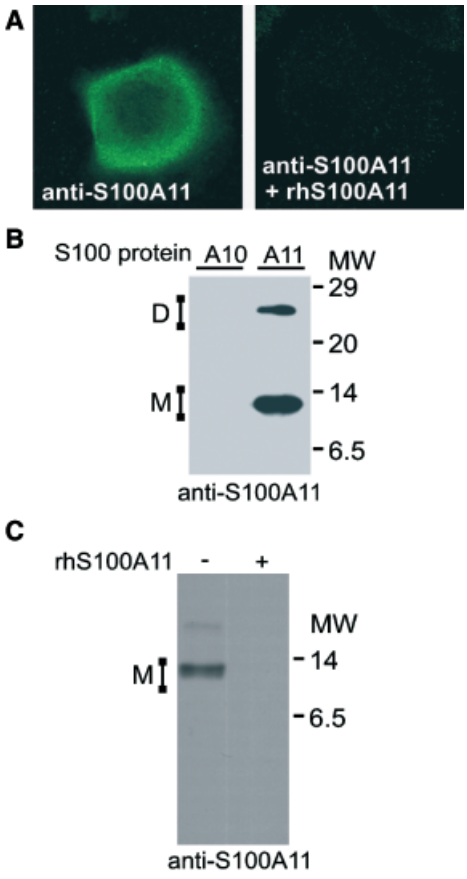

Figure 1
S100A11 antibody specificity. (*A*) Normal human epidermal keratinocytes, grown on coverslips, were fixed and incubated with anti-S100A11 (1:100, 114 μg/mL) without or with adsorption with 340 μg/mL rhS100A11. The slides were then incubated with the appropriate secondary antibody. The fluorescent signal was detected using confocal microscopy. (*B*) Anti-S100A11 does not detect other S100 proteins. Recombinant human S100A10 and S100A11 (5 μg/lane) were electrophoresed and blotted with anti-S100A11. Migration of the S100A11 monomer (*M*) and dimer (*D*) are indicated. (*C*) Detection of S100A11 in keratinocyte total extracts is blocked by incubation with rhS100A11. Total keratinocyte cell extract was prepared, electrophoresed and blotted with anti-S100A11 (1:1000, 11.4 μg/mL) without (–) or with (+) absorption with 34.2 μg per mL rhS100A11. The blot was then incubated with anti-S100A11 and visualized using the appropriate secondary antibody. *M*, migration of the S100A11 monomer.

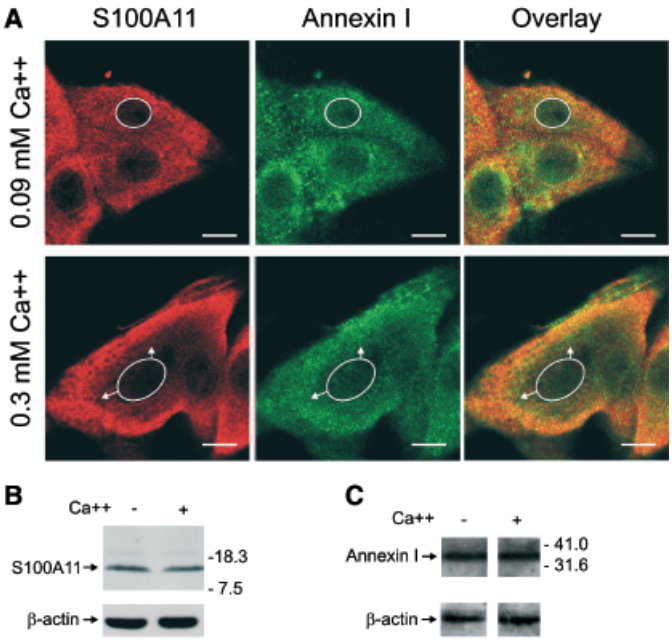

Figure 2
S100A11 colocalizes with annexin I and relocates to the cell periphery upon calcium stimulation. (*A*) Human keratinocytes were cultured on coverslips and treated with low (0.09 mM) or high (0.3 mM) calcium-containing medium for 1 h at 37°C. The cells were then fixed, permeabilized, and stained with anti-S100A11 (*red*) or anti-annexin I (*green*). *Circles*, the perimeter of the nucleus of selected cells. *Arrows*, the direction of redistribution following calcium treatment. The overlay images represent the combined S100A11 and annexin I lineage. *Yellow* indicates colocalization of S100A11 and annexin I. *Bars*, 10 μm. (*B,C*) Calcium treatment does not alter S100A11 or annexin I level. Human keratinocytes were treated as above, and cell extracts were prepared. An equivalent amount of each sample, based on protein, was electrophoresed on a denaturing 12% polyacrylamide gel. The fractionated proteins were then immunoblotted with mouse anti-β-actin, anti-annexin I, or rabbit anti-S100A11. The blots were then washed and incubated with the appropriate secondary detection reagent, and antibody binding was visualized using chemiluminescent detection methods. The numbers indicate molecular weight in kilodaltons.

anti-S100A11 binding to total extracts prepared from keratinocytes.

Intracellular S100A11 localization To identify the intracellular location of S100A11 in cultured keratinocytes, subconfluent cultures, growing in 0.09 mM calcium, were treated with medium containing 0.09 or 0.3 mM calcium for 1 h. The cells were then fixed and costained with anti-S100A11 (*red*) and anti-annexin I (*green*) (Fig 2*A*). The *circles*, in representative cells, indicate the perimeter of the nucleus. In cells maintained in 0.09 mM calcium, S100A11 distributes throughout the cytoplasm, including the region adjacent the nucleus. Annexin I is a known partner of S100A11 (Seemann *et al*, 1996). As expected, annexin I distributes in similar locations. In cells treated with 0.3 mM calcium, S100A11 and annexin I both redistribute toward the cell periphery (*arrows*). Overlay of the red and green images identifies significant signal overlap (*yellow*), suggesting that S100A11 and annexin I partially colocalize. Because the reduction in perinuclear S100A11 and annexin I level observed in calcium-treated cells could be caused by a reduction in protein level, we monitored S100A11 and annexin I protein levels. As shown in Fig 2*B,C*, neither S100A11 nor annexin I level is altered by calcium treatment.

Calcium-dependent S100A11 redistribution requires intact tubulin filaments Tubulin filaments have been reported to mediate S100A11 movement in human glioblastoma cells (Davey *et al*, 2000). We therefore assessed whether this was the case in human keratinocytes. Cells were treated for 2 h with or without 1 μM vincristine, an agent that disrupts and collapses microtubules. The cells were then treated with 0.09 or 0.3 mM calcium for 1 h, fixed, and stained with a cocktail containing anti-S100A11 and anti-β-tubulin (Fig 3). In the absence of vincristine, treatment with 0.3 mM calcium results in a clearing of S100A11 (*green*) away from the nucleus toward the cell periphery (*arrows*). In contrast, treatment with vincristine causes microtubule collapse with formation of perinuclear aggregates (*red*).

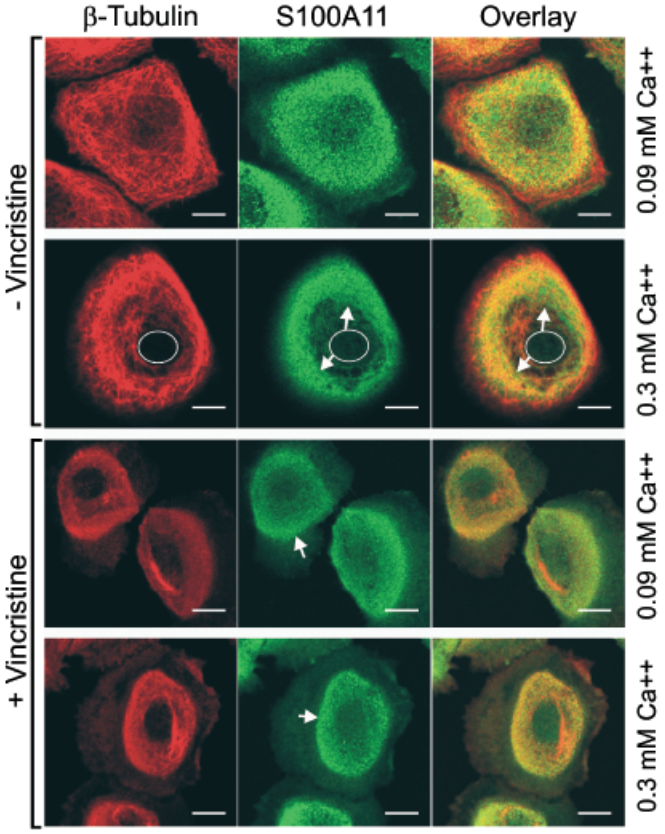

Figure 3
S100A11 redistribution to the cell periphery requires intact tubulin filaments. Keratinocytes, growing on coverslips, were treated for 2 h at 37C in the absence or presence of 1 μM vincristine, a microtubule destabilizer. The cells were then maintained for 1 h in medium containing 0.09 or 0.3 mM calcium, fixed, permeabilized, and costained with anti-S100A11 (*green*) and anti-β-tubulin (*red*). The images were obtained by confocal microscopy. The overlay image indicates the combination of the S100A11 and β-tubulin images. Colocalization is identified by yellow color. *Circles*, the perimeter of the nuclei; *arrows*, indicate the direction of S100A11 redistribution. No arrows are shown in the β-tubulin panel, because β-tubulin does not move in response to calcium treatment. In the vincristine-treated images, the arrows indicate the direction of tubulin retreat from the cell periphery. *Bars*, 10 μm.

S100A11 codistributes to this location (+ *Vincristine, arrows*) and cannot be mobilized to the cell periphery by 0.3 mM calcium. These results suggest that S100A11 interacts with tubulin filaments and that perturbing these filaments influences S100A11 localization.

To assess the specificity of S100A11 interaction with β-tubulin, we assayed for association of a related S100 protein, S100A10, with microtubules. As shown in Fig 4, S100A10 is present in the cytoplasm of cells maintained in medium containing 0.09 mM calcium. Shifting to 0.3 mM calcium does not result in relocation of S100A10 to the cell periphery. In addition, the overlay image indicates that S100A10 does not colocalize with β-tubulin.

To provide direct evidence for S100A11 interaction with β-tubulin, total keratinocyte extracts were precipitated with anti-β-tubulin and blotted with anti-S100A11 or rabbit nonimmune serum. As shown in Fig 5*A*, anti-β-tubulin, but not nonimmune serum, precipitates S100A11. The precipitated S100A11 migrates as a monomer and as additional high-molecular-weight forms (*). Except for one band

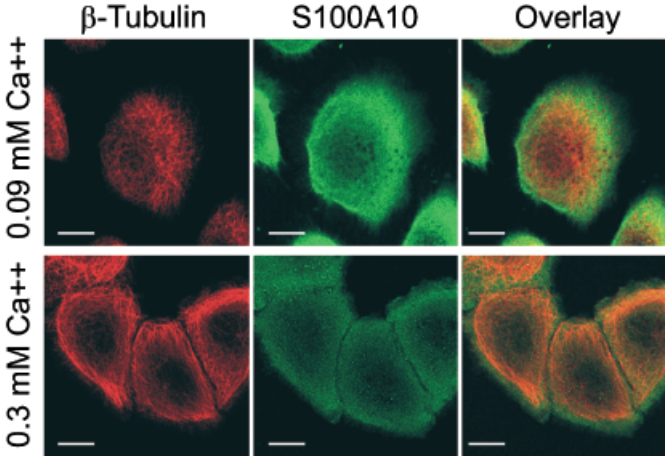

Figure 4
S100A10 does not colocalize with tubulin filaments. Human keratinocytes were cultured on coverslips and treated with low (0.09 mM) or high (0.3 mM) calcium-containing medium for 1 h at 37°C. The cells were then fixed, permeabilized, and immunostained with anti-S100A10 (*green*) or anti-β-tubulin (*red*). The overlay panels indicate the combined images. *Bars*, 10 μm.

migrating at nearly 82 kDa, the monomer and higher molecular weight forms migrate with the corresponding S100A11 forms present in total extract. The inverse experiment is shown in Fig 5*B*. This experiment shows that precipitation of total extracts with anti-S100A11 results in precipitation of β-tubulin.

Because various forms of S100A11 (i.e., monomers, dimers, and high-molecular-weight multimers) exist within the cell and interact with β-tubulin (Fig 5*A*), we examined whether these S100A11 forms are located within specific regions of the cell. Subconfluent keratinocytes were fractioned into a soluble cytosolic fraction, a detergent-soluble membrane fraction, and a detergent-insoluble cytoskeletal fraction. The individual fractions were normalized by volume, electrophoresed, and transferred to nitrocellulose. The blots were then incubated with anti-S100A11 or rabbit nonimmune serum. As show in Fig 5*C*, S100A11 monomers were detected in the cytosolic (supernatant, *S*) fraction. In contrast, high-molecular-weight S100A11-immunoreactive material is observed in the cytoskeletal (pellet, *p*) fraction. No S100A11 immunoreactivity is observed in the solubilized membrane fraction (*M*).

S100A11 does not localize in the Golgi or endoplasmic reticulum (ER) The above results suggest that S100A11 redistributes independently of the Golgi/ER export pathway. To confirm that S100A11 does not localize within the Golgi/ER, we stained keratinocytes with anti-S100A11 and costained with Golgi and ER markers. Figure 6*A* compares the staining pattern of GM130 (*red*), a Golgi matrix protein, and S100A11 (*green*) in cells maintained in a low concentration (0.09 mM) of calcium. As expected, GM130 distributes in a tubular pattern that is particularly intense surrounding the nucleus. In contrast, S100A11 is predominantly localized in the cytoplasm. The overlay indicates that there is no codistribution. Treatment with calcium (0.3 mM) causes S100A11 to move toward the cell periphery (*arrows*), but GM130 remains as a ring surrounding the nucleus. This

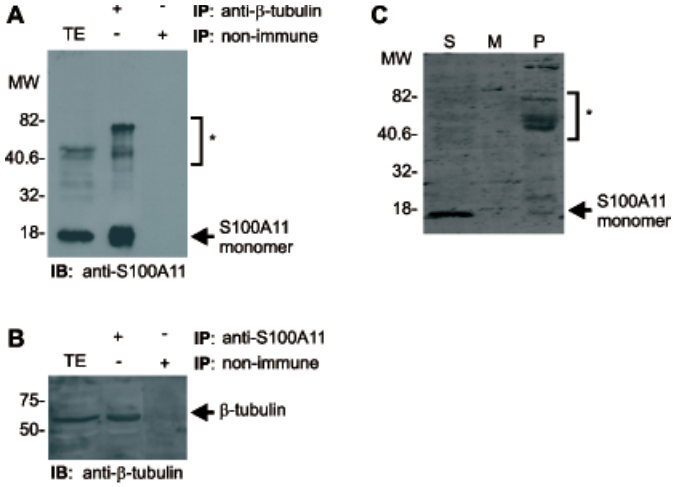

Figure 5
Coprecipitation of S100A11 and β-tubulin. (*A*) Extracts from human keratinocytes grown in 0.09 mM calcium-containing medium were prepared in lysis buffer and then precipitated with anti-β-tubulin or nonimmune serum. The precipitated samples were then electrophoresed and blotted with anti-S100A11. Total extract (*TE*) was electrophoresed in a parallel lane. Migration of the S100A11 monomer, and higher-molecular-weight immunoreactive forms (*) are indicated. (*B*) Extracts, prepared as above, were precipitated using anti-S100A11 or nonimmune serum, electrophoresed, and blotted with anti-β-tubulin. TE was electrophoresed in a parallel lane. Antibody binding was visualized using the appropriate secondary antibody and chemiluminescent detection reagent. (*C*) Cell supernatant, membrane, and particulate fractions were prepared as outlined under Materials and Methods. Equal cell equivalents of each fraction were electrophoresed and immunoblotted with anti-S100A11. The S100A11 monomer is indicated, as is the migration of the larger multimers (*). No signal was observed when nonimmune serum was substituted for anti-S100A11.

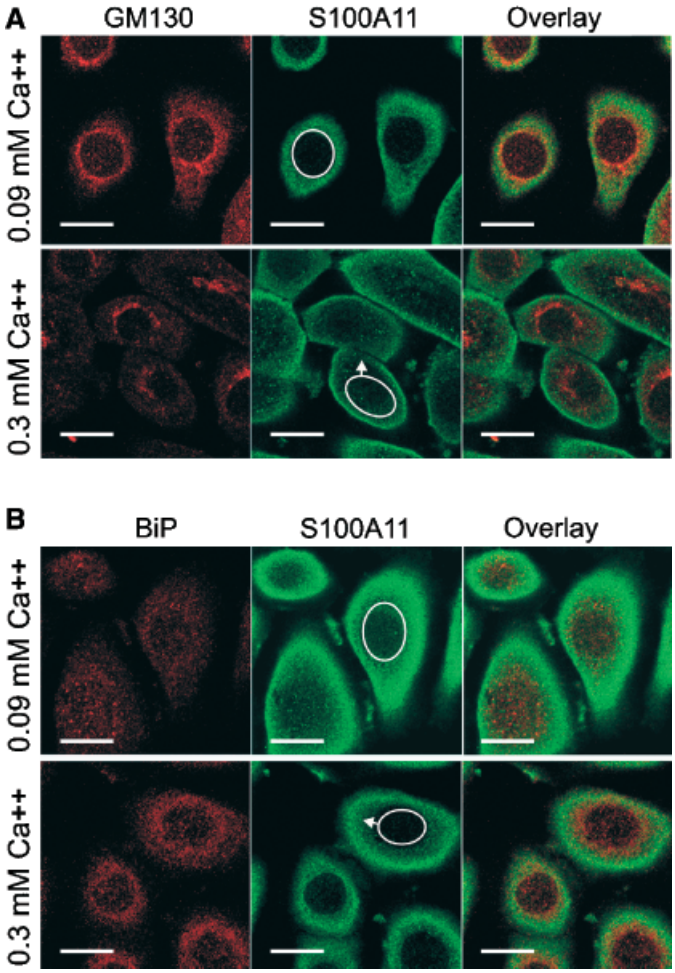

Figure 6
S100A11 does not localize in the ER or Golgi. Keratinocytes, growing on coverslips, were treated for 1 h in medium containing 0.09 or 0.3 mM calcium. The cells were then fixed, permeabilized, and immunostained with a cocktail containing anti-S100A11 (*green*) and either anti-GM130 (*A, red*) or anti-BiP (*B, red*). The images were then collected by confocal microscopy. The overlay image shows the combined red and green signals—no yellow signal is detected, indicating a lack of colocalization of S100A11 and GM130 or BiP. *Bars*, 10 μm.

is particularly evident in the overlay (Fig 6*A*) where the S100A11-associated green stain is in the cell periphery and the GM130-associated red stain decorates the nuclear perimeter.

Figure 6*B* compares the distribution of the ER marker BiP and S100A11. BiP staining (*red*) is present in a punctate pattern surrounding the nucleus. In comparison, S100A11 is present throughout the cytoplasm. Treatment with calcium causes S100A11, but not BiP, to clear from the perinuclear region (*arrows*). This is best visualized by comparing the intermingling of the BiP-positive structures and the S100A11 staining in the overlay of cells maintained in 0.09 mM calcium to the distinct separation of the BiP and S100A11 staining in the overlay of cells treated with 0.3 mM calcium.

Brefeldin A disruption of the Golgi complex does not alter S100A11 distribution Brefeldin A is a fungal metabolite that causes disintegration of the Golgi complex and interferes with classical protein secretion by blocking vesicle budding in the secretory pathway. Brefeldin A-treated Golgi form elongated tubules along the cell periphery (Sciaky *et al*, 1997) that eventually fuse with the ER, blocking retrograde transport and protein secretion (Pelham, 2001). In the absence of Brefeldin A treatment, the Golgi complex, as visualized using anti-GM130 (*red*), forms a loosely oriented array around the nucleus (Fig 7*A*). Disruption of the Golgi apparatus, as evidenced by the redistribution of GM130 staining, occurs in keratinocytes after 30 min of brefeldin A treatment (Fig 7*A*). Figure 7*B* (– brefeldin A) shows the redistribution of S100A11 that occurs following calcium treatment (*left panels, arrows*). The *right panels* show that brefeldin A treatment does not impede the calcium-dependent redistribution of S100A11 to the cell periphery (*arrows*). These results suggest that S100A11 distribution and redistribution is not mediated by the Golgi/ER system.

S100A11 in epidermal keratinocytes *in vivo* Taken together, the above results suggest that S100A11 redistributes to the cell periphery in calcium-treated keratinocytes and predicts that S100A11 may associate with peripheral structures in keratinocytes *in vivo*. To examine the *in vivo* distribution of S100A11, sections of normal and psoriatic epidermis were stained with anti-S100A11. Figure 8 displays the peripheral staining of S100A11 that is apparent in suprabasal epidermal keratinocytes. This peripheral staining is observed in both normal and psoriatic epidermis (*arrows*). In a small percentage of cells there is intense nuclear staining (*circles*).

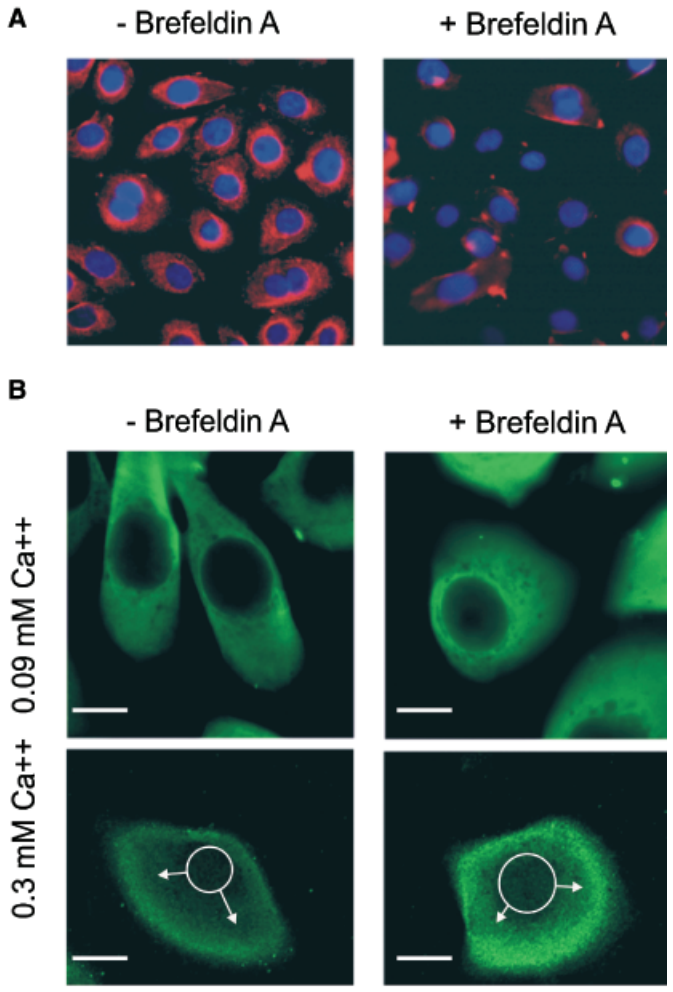

Figure 7
Redistribution of S100A11 does not require an intact Golgi complex. (*A*) Keratinocytes, grown on coverslips, were fixed, permeabilized, and treated for 30 min in the presence or absence of 5 μg per mL brefeldin A, an agent that disrupts the Golgi apparatus. The cells were then collected, fixed, and incubated with anti-GM130 (*red*) and stained with Hoechst nuclear stain (*blue*). (B) Cells were treated without or with brefeldin A for 30 min and then further treated with 0.09 or 0.3 mM calcium for 1 h at 37°C followed by immunostaining with anti-S100A11 (*green*). *Arrows*, redistribution of S100A11 in response to calcium treatment. *Circles*, the perimeter of the nuclei. *Bars*, 10 μm.

As noted above, nuclear localization was not observed in subconfluent cultured keratinocytes. One possibility is that the extent of confluence may promote nuclear appearance of S100A11. To evaluate this, we maintained postconfluent cultures for several days and then compared the effects of calcium treatment on S100A11 distribution. As shown in Fig 9*A*, in confluent cultures maintained in 0.09 mM calcium, S100A11 is distributed in the cytoplasm with no nuclear staining. The cytoplasmic distribution is particularly evident in the orthogonal image (*yellow arrow*). The confocal series, shown at the *right*, indicates the absence of nuclear staining in successive optical sections (1.8 μm is near the substrate/cell interface, whereas the 12.23-μm section is near the culture surface). Figure 9*B* shows that after a 1-h treatment in 0.3 mM calcium-containing medium, S100A11 has moved to the cell periphery as indicated by the *white arrows* (top view) and the *yellow arrow* (orthogonal view).

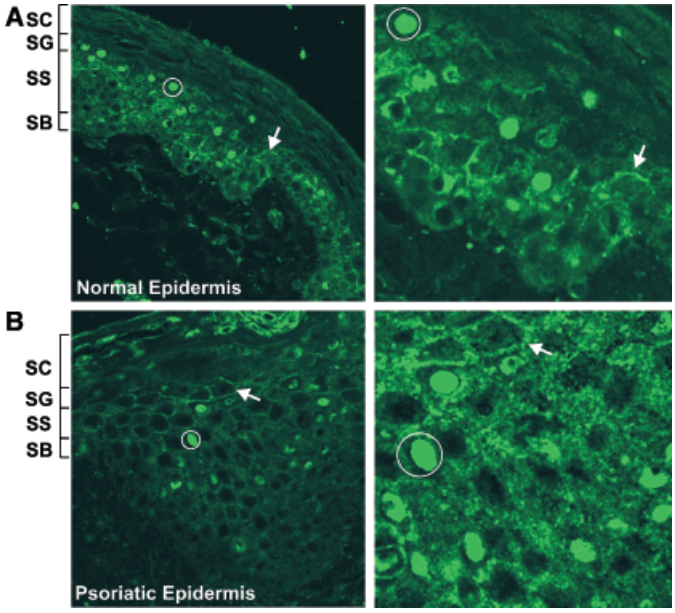

Figure 8
S100A11 in epidermis. Biopsies were excised from normal and psoriatic-involved epidermis. The specimens were fixed, processed, and stained with rabbit anti-human S100A11 (diluted 1:100). (*A*) Normal epidermis stained with anti-S100A11. The *right panel* is a higher magnification image of the *left panel*. (*B*) Involved psoriatic epidermis stained with anti-S100A11. The *right panel* is a higher magnification image of the *left panel*. S100A11 is distributed in the cytoplasm of cells in the basal and spinous layers. The *arrows* indicate peripheral S100A11 localization in the granular layer. Some nuclear staining is observed in selected upper granular layer cells (circles). Parallel sections stained with preimmune serum showed no reactivity (not shown).

Again, no S100A11 is detected within the nucleus. In addition, S100A11 redistribution after treatment for 1 h with 1.2 mM calcium-containing medium was not significantly different from the S100A11 redistribution observed after 0.3 mM calcium treatment (not shown).

Nuclear staining of Langerhans cells An alternate possibility is that the nuclear staining (Fig 8) identifies another epidermal cell type. The epidermis harbors a limited population of dendritic antigen-presenting cells—the Langerhans cells (Nestle and Nickoloff, 1995). To assess whether Langerhans cells express nuclear S100A11, epidermal sections, prepared from normal and psoriatic tissue, were stained with anti-S100A11 and anti-CD1a. CD1a is a cytoplasmic Langerhans cell marker (Teunissen, 1992). Figure 10*A* shows the peripheral green anti-S100A11 staining in keratinocytes (*arrows*). Staining of normal epidermis with anti-CD1a specifies a small number of Langerhans cells (e.g., *circle*). More CD1a-positive cells are present in this section of psoriatic epidermis. It is interesting that many of the CD1a-positive cells also display nuclear anti-S100A11 immunoreactivity (e.g., *circle*). Figure 10*B* shows an enlargement of the boxed area from Fig 10*A*. This S100A11/CD1a-positive cell shows the cytosolic staining characteristic of CD1a (*red*) and the nuclear staining of S100A11 (*green*). These results suggest that the S100A11 nuclear epidermal staining is due to S100A11 presence in the nucleus of Langerhans cells.

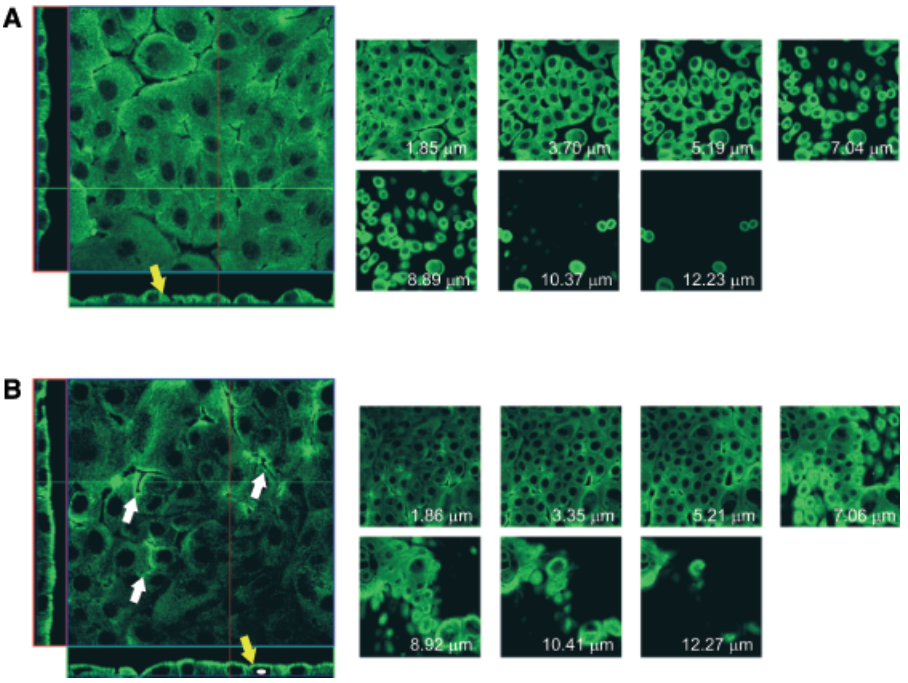

Figure 9
S10011 distribution and redistribution in confluent keratinocytes. Keratinocytes were plated on coverslips and permitted to become confluent. (*A*) Keratinocytes, maintained at confluence for several days in medium containing 0.09 mM calcium, were harvested, fixed, stained, and incubated with anti-S100A11 (diluted 1:100). The small frames indicate serial images beginning near the cell/substrate interface (1.85 μm) and proceeding upward to the cell surface (12.23 μm). The large image includes orthogonal sections. *Yellow arrow*, S100A11 staining in the cytoplasm. (*B*) Cells, maintained at confluence for several days in medium containing 0.09 mM calcium, were treated with 0.3 mM calcium for 1 h at 37°C. The cells were harvested, fixed, and incubated with anti-S100A11 (diluted 1:100). The small frames indicate serial images beginning near the cell/substrate interface (1.86 μm) and proceeding upward to the cell surface (12.27 μm). *Yellow arrow*, peripheral S100A11 staining on the orthogonal section. *White oval*, indicates the extent of the nucleus. *White arrow*, also indicates the peripheral S100A11 staining in the surface image.

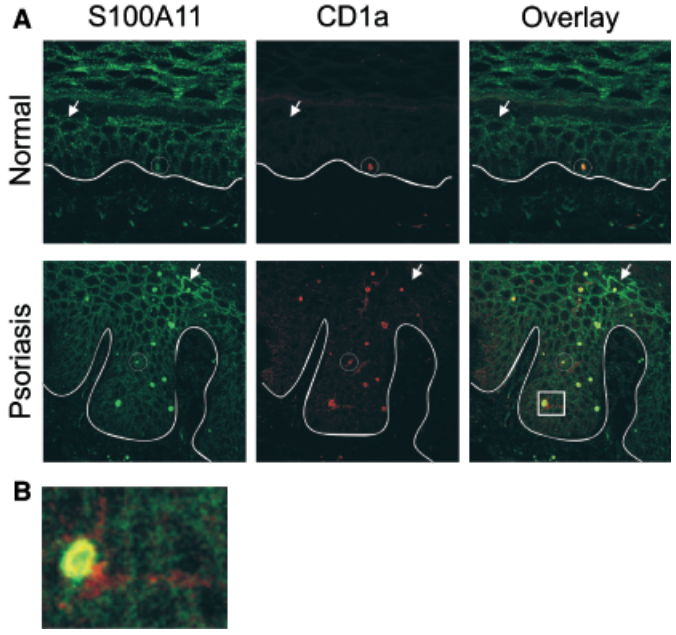

Figure 10
Nuclear staining of S100A11 in Langerhans cells. (*A*) Normal and psoriatic epidermal sections were costained with 1:100 diluted rabbit anti-human S100A11 (*green*) and 1:100 diluted mouse anti-CD1a (*red*). Sections stained in the absence of primary antibody showed no staining (not shown). *Arrows*, peripheral S100A11 staining in keratinocytes; *circles*, a representative CD1a-positive cell; *line*, traces the dermal/epidermal junction. (*B*) This panel shows an enlarged view of the section of *A* enclosed by the rectangle. The Langerhans cell dendrites are indicated by the projections.

S100A11 peripheral distribution is enhanced by 12-*O*-tetradecanoylphorbol-13-acetate and thapsigargin The above studies show that treatment of keratinocytes with calcium promotes S100A11 redistribution to the cell periphery. To determine whether other keratinocyte regulatory agents (Efimova *et al*, 1998; Balasubramanian *et al*, 2000) produce a similar response, cells were treated with 12-*O*-tetradecanoylphorbol-13-acetate or thapsigargin for 60 min. Figure 11 shows that both of these agents promote peripheral redistribution of S100A11 immunoreactivity.

Discussion

Calcium- and microtubule-dependent redistribution of S100A11 S100 proteins comprise a family of structurally related proteins that serve as markers of cell proliferation, death, and differentiation (Donato, 1999; Heizmann and Cox, 1998). Subcellular distribution and stimulus-dependent redistribution of S100 protein complexes are important factors that influence S100 protein activity and function. In keratinocytes, S100 proteins are transglutaminase substrates and cornified envelope precursors (Robinson *et al*, 1997; Robinson and Eckert, 1998; Ruse *et al*, 2001). Thus, S100 proteins serve as models for studying the mechanism whereby soluble proteins are incorporated into the cornified envelope. In this study, we examine the distribution of S100A11 in keratinocytes and its redistribution following treatment with differentiating agent.

In glioblastoma cells, S100 proteins undergo calcium-dependent translocation from the cytosol to plasma membrane-associated vesicles (Davey *et al*, 2000). Our studies show that elevating extracellular calcium results in a net redistribution of S100A11 from the cytoplasm toward the cell periphery in keratinocytes. This redistribution is inhibited by vincristine, a microtubule disassembly agent. Vincristine promotes tubule collapse and the resulting formation of a perinuclear tubulin ring. S100A11 redistributes to this ring, presumably carried to this location attached to tubulin. Immunoprecipitation experiments confirm an S100A11 interaction with tubulin. In contrast, S100A11 does not localize with Golgi or ER markers, suggesting that S100A11 does not distribute in these structures. In addition, brefeldin A,

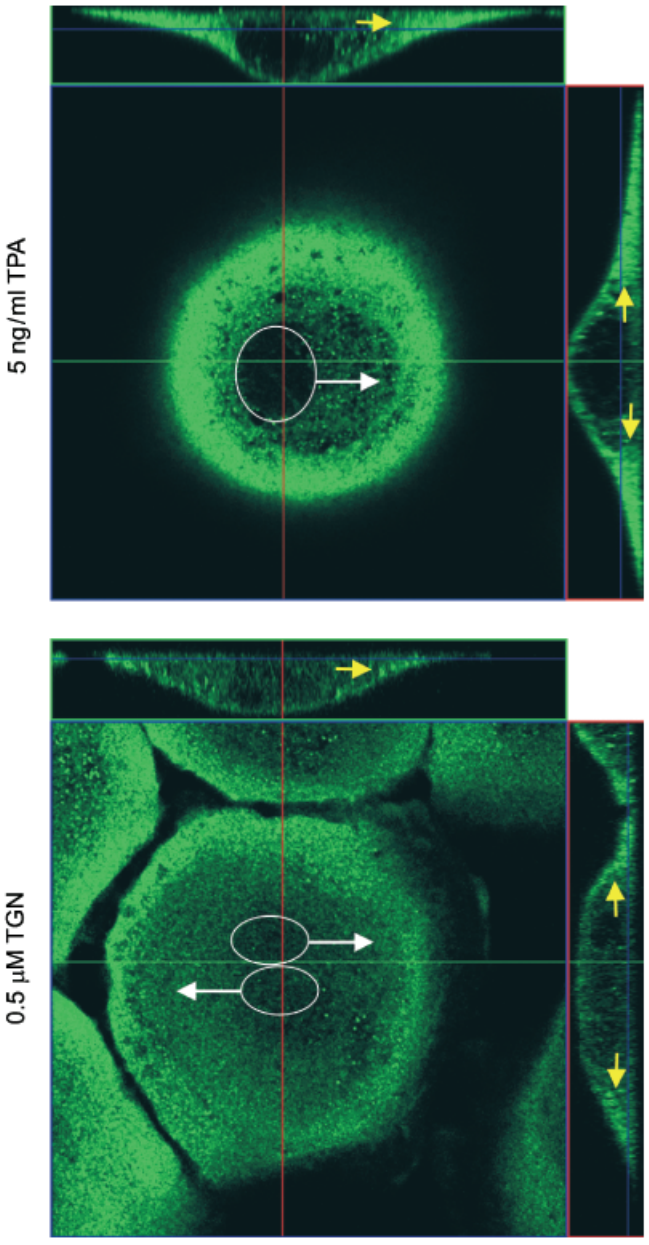

Figure 11
Regulation of S100A11 distribution by other regulatory agents. Keratinocytes, growing on coverslips, were treated for 1 h with 5 ng per mL 12-*O*-tetradecanoylphorbol-13-acetate or 0.5 μM thapsigargin (*TGN*). The cells were then fixed, permeabilized, and immunostained with rabbit anti-human S100A11 (*green*). *White arrows*, redistribution of S100A11 in response to treatment with differentiating agents, 12-*O*-tetradecanoylphorbol-13-acetate or TGN. *Circles*, the perimeter of the nuclei. *Yellow arrows*, the direction of S100A11 redistribution in the orthogonal sections.

which blocks the classical ER–Golgi secretory pathway, does not influence calcium-dependent S100A11 redistribution or localization, suggesting that the classical secretory pathway is not involved in S100A11 redistribution. Our results in keratinocytes agree with another published report wherein Heizmann and colleagues observed Brefeldin A-independent redistribution of S100A11 in glioblastoma cells (Davey *et al*, 2000). These results suggest that S100A11 associates with microtubules and that microtubules mediate movement of S100A11 to the cell periphery.

A similar dependence on microtubules has been noted for other S100 proteins. Rammes *et al* (1997) noted that S100A8 and S100A9 are secreted independently of the classic Golgi/ER pathway and Roth and coworkers (1993) demonstrated a microtubule requirement for S100A8/S100A9 redistribution in myelomonocytic cells. Our studies further suggest that S100A11 remains associated with microtubules following brefeldin A-associated collapse. This is consistent with the observations of Donato and colleagues who showed that S100B remains associated with microtubules following treatment with agents that alter microtubule structure in U251 glial cells (Sorci *et al*, 1998). Taken together, these results suggest that S100 proteins migrate on microtubules. The absence of movement via a Golgi/ER mechanism is consistent with the observation that S100 proteins lack the signal sequences (Schafer and Heizmann, 1996) required for secretion. This Golgi/ER-independent mechanism has been noted for a limited set of proteins and was first described for cytokine secretion by Muesch *et al* (1990).

Heizmann and colleagues described S100A11 movement to the cell periphery coupled with formation of S100A11-enriched cell surface vesicles in glioblastoma cells (Davey *et al*, 2000). In contrast, in human keratinocytes, we observe distribution of S100A11 to the cell periphery without vesicle formation. This could result from the use of a different stimulus, as Heizmann and coworkers stimulated with thapsigargin (Davey *et al*, 2000), an agent that promotes the release of intracellular calcium stores via depletion of IP_3-senstive ER stores, and we stimulated with increased extracellular calcium, a physiologic stimulus of keratinocyte differentiation (Rice and Green, 1979; Boyce and Ham, 1983; Hennings *et al*, 1989; Eckert *et al*, 1997). Nevertheless, additional studies in keratinocytes (Fig 11) reveal that thapsigargin and 12-*O*-tetradecanoylphorbol-13-acetate produce a redistribution to the cell periphery that is essentially identical to that observed with calcium. This suggests that treatment with thapsigargin causes S100A11 to move via different mechanisms and to different locations in the two cell types. It also implies that S100A11 function may differ in the two cell types.

S100A11 colocalizes with annexin I Gerke and colleagues have shown that the calcium/phospholipid-binding protein annexin I forms a complex with S100A11 (Seemann *et al*, 1996). *In vitro*, this binding inhibits the protein kinase C-associated phosphorylation of annexin I (Naka *et al*, 1994), suggesting that S100A11 may influence protein kinase C-dependent signal transduction. We monitored S100A11 and annexin I distribution before and following calcium treatment and found a strong colocalization of S100A11 and annexin I and the redistribution of both proteins to a peripheral location following calcium treatment. We hypothesize that this movement is necessary to position S100A11 and annexin I for transglutaminase-dependent incorporation into the cornified envelope (Robinson *et al*, 1997; Robinson and Eckert, 1998; Ruse *et al*, 2001). Type I transglutaminase is a plasma membrane-anchored enzyme in keratinocytes that catalyzes formation of covalent isopeptide bonds and drives assembly of the cornified envelope (Phillips *et al*, 1993; Rice *et al*, 1992). Our

results suggest that S100A11 and annexin I are delivered from the cell cytosol to the cell periphery via a calcium- and microtubule-dependent mechanism and that both S100A11 and annexin I are then crosslinked by type I transglutaminase, a calcium-activated enzyme, to form part of the cornified envelope. Thus, the present studies suggest a tubulin-associated mechanism whereby cornified envelope precursors can be delivered to the site of crosslink formation. This is consistent with the finding in Fig 5 that the particulate fraction contains high-molecular-weight anti-S100A11-immunoreactive bands. It is possible that these bands represent partially crosslinked S100A11 (Robinson *et al*, 1997; Robinson and Eckert, 1998; Ruse *et al*, 2001; Broome *et al*, 2003). It is intriguing that the material is found in the particulate/cytoskeletal fraction and that no S100A11 form was found associated with the membranous fraction.

Distribution of S100A11 in epidermis Based on our results using calcium-treated cultured keratinocytes, we anticipated a peripheral distribution of S100A11 in differentiated epidermis *in vivo*. Indeed, most cells in the epidermal granular layer display a peripheral, membrane-associated S100A11 localization in both normal and psoriatic epidermis. Nevertheless, in addition to peripheral staining, selected cells in the upper granular layer in normal epidermis and throughout the epidermis in psoriasis displayed nuclear staining. This is a particularly interesting result, considering that Sakaguchi *et al* (2000) described nuclear localization of phosphorylated S100A11 in confluent immortalized human fibroblasts. This localization was associated with elevation of p16 and p21 expression (Sakaguchi *et al*, 2000). Elder and colleagues have also shown that another S100 protein, S100A2, can shuttle between the nucleus and cytoplasm in a stimulus-dependent manner (Zhang *et al*, 2002). This finding suggests that S100 proteins can move to the nuclear compartment in some cell types. We could not detect nuclear S100A11 staining in these cultures, indicating that nuclear presence is a rare event in cultured cells, at least in our culture system. Because nuclear staining was restricted to a few cells in epidermis, we suspected that the nuclear stained cells may identify a nonkeratinocyte cell type. Langerhans cells comprise a population of epidermal antigen-presenting cells (Teunissen, 1992; Cumberbatch *et al*, 2000). Langerhans cell precursors arise from CD34 + monocytes in the bone marrow and circulate in the blood before localizing in the skin. CD1a is not expressed in the precursor cells, but is expressed in epidermal-localized Langerhans cells (Banchereau *et al*, 2003; Reynolds *et al*, 1995; Vincent *et al*, 2002; Vincent e*t* al, 2003). Our studies suggest that S100A11 is present in the nucleus of CD1a-positive Langerhans cells and that most, perhaps all, of the S100A11 nuclear staining in epidermis is accounted for by this population of cells. Additional studies, revealing an absence of CD14 staining (Vincent *et al*, 2002; Vincent *et al*, 2003), confirm that these cells are not peripheral blood monocytes or tissue macrophages (not shown). Langerhans cells are known to express S100B (Boni *et al*, 1997); however, expression of other S100 proteins has not been assessed. The present studies suggest that Langerhans cells also express S100A11. This may not be surprising, because S100A11 and S100B are known to colocalize in U373 astrocytoma cells and S100B and S100A11 form calcium- and zinc-dependent heterocomplexes (Deloulme *et al*, 2000). Thus, S100A11 and S100B may participate in an at present unknown manner to regulate Langerhans cell function.

In summary, based on the findings presented in this article and those of previous reports (Robinson *et al*, 1997; Robinson and Eckert, 1998; Ruse *et al*, 2001; Broome *et al*, 2003), we speculate that S100A11 is present in the cytoplasm of resting keratinocytes and, in response to an increase in extracellular calcium, S100A11, along with annexin I, moves to the plasma membrane where it performs a presently unknown signaling function before being incorporated, via a type I transglutaminase-dependent mechanism, as a component of the keratinocyte cornified envelope. These findings suggest a mechanism of envelope precursor redistribution to plasma membrane crosslinking sites that involves transport along microtubules.

Materials and Methods

Keratinocyte cell culture Passage 3 human foreskin keratinocytes were maintained in keratinocyte serum free-medium, containing 0.09 mM calcium, and supplemented with bovine pituitary extract and epidermal growth factor (Efimova *et al*, 1998). In some experiments, keratinocytes were transferred to medium containing the differentiating agent, calcium, at 0.3 mM, for 1 h before harvest.

Antibody to recombinant human S100A11 Recombinant human S100A11 protein was produced in *Escherichia coli* (BL21 DE3) (Ruse *et al*, 2001). Bacteria were transformed with pET28a + S100A11, which encodes a polyhistidine–S100A11 fusion protein, and His–S100A11 production was induced by addition of isopropylthio-β-D-galactoside. Following protein purification by chromatography on a His-Bind column (Novagen), the polyhistidine track was removed by cleavage with thrombin, and the cleaved S100A11 protein was removed from the polyhistidine fragment by His-Bind column chromatography. Recombinant human S100A11 protein retains a three-amino-acid extension (Gly-Ser-His) at the amino terminus. Rabbit anti-human S100A11 antibody was prepared by immunizing rabbits with recombinant human S100A11 (1 mg of S100A11 per animal) three times over a period of 2 mo. At 4 mo, immune serum was collected from each rabbit, and the IgG fraction was isolated by a salting-out procedure (50% ammonium sulfate). We confirmed that the anti-S100A11 antibody reacts specifically with the human S100A11 protein by immunoblot. The optimal antibody concentration for immunofluorescence (1:1000) and immunoblot (1:1000) was determined by serial dilution (Broome *et al*, 2003).

Antibodies and reagents The antibodies include goat anti-human annexin I (sc1922, Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-human BiP (610978, BD Transduction Laboratories, San Diego, CA), mouse anti-human GM130 (610822, BD Transduction Laboratories), mouse anti-β-tubulin (T4026, Sigma, St. Louis, MO), mouse anti-β-actin (A5441, Sigma), mouse anti-CD1a (sc5265, Santa Cruz Biotechnology), mouse anti-CD14 (C7673, Sigma), Alexa Fluor 488-conjugated goat anti-rabbit IgG (A11008, Molecular Probes, Eugene, OR), Alexa Fluor 488-conjugated donkey anti-goat IgG (A11055, Molecular Probes), Cy3-conjugated sheep anti-mouse IgG (C2181, Sigma), Cy3-conjugated sheep anti-rabbit IgG (C2306, Sigma), horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG (NA934,

Amersham Pharmacia Biotech, Piscataway, NJ), and biotin-labeled goat anti-rabbit IgG (PK-6101, Vector Laboratories, Burlingame, CA). Vincristine (V8879, Sigma) was dissolved in deionized water as a 1 mM stock solution. Brefeldin A (203729, Calbiochem, La Jolla, CA) was prepared as a 10 μg per mL stock in methanol.

Protein immunoblots Equivalent quantities of protein (50 μg/lane), prepared from rapidly frozen keratome samples or lyzed cultured human keratinocytes, were electrophoresed on denaturing and reducing 12% polyacrylamide gels, and the fractionated proteins were transferred to nitrocellulose. Membranes were blocked with 1% bovine serum albumin for 1 h at room temperature with gentle shaking, washed, and incubated for 1 h at 25°C with primary antibody. After being washed, the membrane was incubated for 1 h at 25°C with HRP-conjugated secondary antibody (diluted 1:10,000). After being washed, the blots were immersed in ECL western blotting detection reagent (Amersham Pharmacia Biotech) for 1 min. Chemiluminescence was visualized using Kodak X-OMAT AR imaging film (Eastman Kodak, Rochester, NY). β-actin was detected using mouse anti-β-actin (1:1000) followed by HRP-conjugated sheep anti-mouse IgG.

Cellular fractionation Near-confluent keratinocyte cultures (6×50-cm^2 dishes) were washed with phosphate-buffered saline (PBS) and scraped into 1 mL of cell homogenization buffer (20 mM Hepes, 2 mM ethylenediaminetetraacetic acid, 15.5 ng dithiothreitol, 1 mM phenylmethylsulfonyl fluoride). Cells were disrupted by sonication, centrifuged at 16110 g at 4°C, and the supernatant was saved as the soluble, cytosolic fraction. The cell pellet was resuspended in 1 mL of cell homogenization buffer plus 5% Triton, sonicated, and centrifuged at 16110 g at 4°C. The resulting supernatant was collected as the detergent-soluble, membrane fraction. The remaining pellet was reconstituted in Laemmli sample buffer. The fractioned proteins were electrophoresed on a 12% acrylamide gel, and transferred to polyvinylidene difluoride membrane. The blots were incubated overnight with polyclonal rabbit anti-S100A11 (1:1000), washed, and incubated for 1 h with HRP-conjugated donkey anti-rabbit IgG (1:10,000). The blots were washed, after incubation with the secondary antibody, and exposed to ECL detection reagents.

Tissue immunofluorescence Keratomes, excised from normal and psoriatic patients, were fixed for 12 h at 4°C in 2% paraformaldehyde, processed, sectioned, deparaffinized, ethanol-rehydrated, and rinsed with PBS. Nonspecific antibody binding was blocked by incubating the sections for 30 min at 25°C in 1.5% rabbit serum (S-5000, Vector Laboratories) in PBS. Blocked sections were incubated for 1 h at 25°C with rabbit anti-S100A11 or mouse anti-CD1a diluted 1:100 in PBS. The sections were then rinsed three times with PBS and incubated for 1 h at 25°C with Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:1000), a sensitive fluorescence detection reagent, or Cy3-conjugated sheep anti-mouse IgG (1:1000). The slides were examined by a laser scanning confocal microscope (Zeiss LSM510, Thornwood, NY) with a $63 \times$ N.A. 1.4 oil immersion plan-apochromat objective. Confocal images of green fluorescence were collected using 488-nm excitation light from an argon/krypton laser, a 488-nm dichroic mirror, and a 500- to 550-nm bandpass barrier filter. Images of red fluorescence were collected using a 543-nm excitation light from the HeNeI laser, a 543-nm dichroic mirror, and a 560-nm long pass filter. The images were analyzed and processed using Adobe Photoshop (version 7.0). The images are representative of at least three separate experiments in which five fields were examined.

Immunofluorescence of cultured cells Human keratinocytes were plated onto 22×22-mm coverslips. After 24 h, cells were treated with either 0.09 mM calcium chloride (low calcium) or 0.3 mM calcium chloride (high calcium) for 1 h. The cells were then fixed with 4% paraformaldehyde for 1 h, permeabilized with methanol for 10 min, and incubated with a primary antibody cocktail containing rabbit anti-human S100A11 (1:1000) and goat anti-human annexin I (1:500), mouse anti-β-tubulin (1:5000), mouse anti-human BiP (1:250), or mouse anti-human GM130 (1:250). The secondary antibody cocktail contained the appropriate combination of Alexa Fluor 488-conjugated anti-rabbit IgG (1:1000), Cy3-conjugated sheep anti-mouse-IgG (1:500), Alexa Fluor 488-conjugated donkey anti-goat IgG (1:1000), or Cy3-conjugated sheep anti-rabbit-IgG (1:500). The coverslips were then sealed onto microscope slides using DABCO antifade reagent and sealant (Molecular Probes). The slides were then examined by scanning confocal microscopy, and the images were analyzed and processed using Adobe Photoshop (version 7.0). The images are representative of at least three separate experiments in which five fields were examined.

Immunoprecipitation Near-confluent keratinocyte cultures (6×50-cm^2 dishes) were washed with PBS and scraped into 1 mL of cell lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 1 mM ethylene glycol bis tetraacetic acid, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na_3VO_4, 1 μg per mL leupeptin, 1 mM phenylmethylsulfonyl fluoride). Cells were disrupted by sonication and centrifuged at $14,000 \times g$ at 4°C, and the supernatant was saved as whole-cell lysate. Protein concentrations were determined by Bradford assay.

Total protein (500 μg) was incubated with 2 μg of mouse anti-β-tubulin antibody (sc5274, Santa Cruz Biotechnology) or 1 μg of rabbit anti-S100A11 antibody for 3 h at 4°C. Protein–antibody complexes were then precipitated with protein (A + G)–agarose (sc2003, Santa Cruz Biotechnology) for 1 h at 4°C. Precipitates were washed three times with lysis buffer and resuspended in 50 μL Laemmli sample buffer. The precipitated proteins were electrophoresed, in parallel, with 50 μg of whole cell lysate on a 12% acrylamide gel and transferred to polyvinylidene difluoride membrane. For β-tubulin detection, the blots were incubated overnight with mouse anti-β-tubulin antibody (1:1000, T4026, Sigma), washed, and incubated with HRP-conjugated donkey anti-mouse IgG (1:10,000) for 1 h. For detection of S100A11, the blots were incubated overnight with polyclonal rabbit anti-S100A11 (1:1000), washed, and incubated for 1 h with HRP-conjugated donkey anti-rabbit IgG (1:10,000). All blots were washed after incubation with the secondary antibody and exposed to ECL detection reagents.

Microtubule disruption Human keratinocytes were plated onto 22×22-mm coverslips. After 24 h, cells were treated with or without 1 μM vincristine for 2 h at 37°C, to disrupt the microtubule network, and then stimulated with either 0.09 or 0.3 mM calcium chloride for 1 h. The cells were fixed with 4% paraformaldehyde for 1 h, permeabilized with methanol for 10 min, and incubated with a primary antibody cocktail containing rabbit anti-human S100A11 (1:1000) and mouse anti-β-tubulin (1:5000). The secondary antibody cocktail contained Alexa Fluor 488-conjugated goat anti-rabbit-IgG (1:1000) and Cy3-conjugated sheep anti-mouse-IgG (1:500). The coverslips were sealed onto microscope slides and examined by confocal microscopy.

This work utilized the facilities of the Skin Diseases Research Center of Northeast Ohio (NIH AR39750) and the Ireland Cancer Center Confocal Facility (NIH P30CA43703) and was supported by grants from the National Institutes of Health (R.L.E.). The Developmental Biology Training Program (NIH HD07104-25) provided A.M.B.'s salary.

DOI: 10.1046/j.0022-202X.2003.22105.x

Manuscript received February 14, 2003; revised August 20, 2003; accepted for publication September 4, 2003

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